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Assessment of the Proliferative Activity of Superficial Esophageal Carcinoma Using MIB-1 Immunostaining for the Ki-67 Antigen

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Background and Objectives: Lymph node metastasis or vascular invasion may occur in superficial esophageal squamous cell carcinoma when it invades to or into the muscularis mucosae. Therefore, the correlation between histopathological characteristics and the proliferative activity of superficial esophageal carcinoma was investigated.
Methods: Thirty-eight cases of esophageal squamous cell carcinoma, including 14 cases of mucosal carcinoma and 24 cases of submucosal carcinoma, who underwent surgical resection without preoperative treatment, were studied using monoclonal antibody MIB-1 for the Ki-67 antigen immunohistochemically. The labeling index (LI) was calculated with a computed image analyzer.
Results: The LI of MIB-1 at the invasive tip of m_3 carcinoma was significantly higher than that of m_1 or m_2 carcinoma ($P < 0.01$). The LI at the invasive tip was significantly higher than that at the core of sm_2 ($P < 0.05$) and submucosal carcinoma overall ($P < 0.01$). The LI values at both the invasive tip and core of poorly differentiated carcinoma in submucosal carcinoma were higher than that of well or moderately differentiated carcinoma with a significant difference ($P < 0.05$). The LI at the invasive tip of submucosal carcinoma with lymph node metastasis or lymphatic invasion was significantly higher than that without them ($P < 0.05$).
Conclusion: Proliferative activities of cancer cells in superficial esophageal squamous cell carcinoma, immunostaining with the MIB-1, were related to the depth of invasion, differentiation, lymph node metastasis, and lymphatic invasion with a significant difference.
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KEY WORDS: superficial carcinoma of the esophagus; squamous cell carcinoma; proliferative activity; immunohistochemicals study; monoclonal antibody MIB-1

INTRODUCTION

Detection of superficial esophageal carcinoma has increased along with the progress in diagnostic techniques [1,2]. In Japan, it has been proposed that mucosal and submucosal esophageal carcinoma should be classified by the depth of invasion into the following six types: m_1, carcinoma confined to the epithelium or extending slightly beyond the basement membrane; m_2, carcinoma invading the lamina propria mucosae but not the muscularis mucosae; m_3, carcinoma in contact with the muscularis mucosae; sm_1, carcinoma minimally invading the

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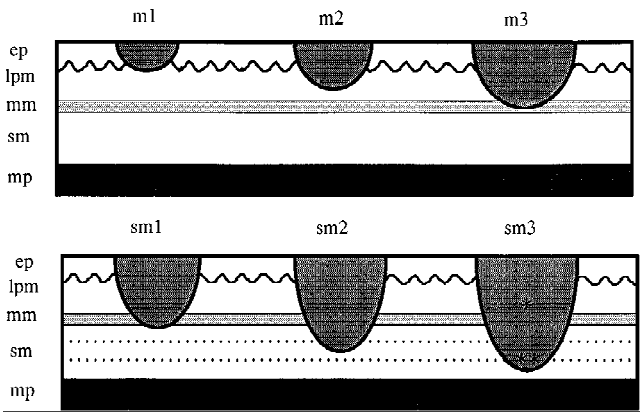

Fig. 1. Superficial esophageal carcinoma: subclassification of depth of invasion. *Core of the tumor. **Invasive tip of the tumor. ep, epithelium; lpm, lamina propria mucosae; mm, muscularis mucosae; sm, submucosa; mp, muscularis propria. m_1, intraepithelial carcinoma; m_2, carcinoma limited to the proper mucosal layer; m_3, carcinoma invaded to the muscularis mucosae; sm_1, carcinoma limited to the upper one third of the submucosal layer; sm_2, carcinoma limited to the middle one third of the submucosal layer; sm_3, carcinoma limited to the lower one third of the submucosal layer.

upper submucosa; sm_2, carcinoma with definite invasion of the submucosa; sm_3, carcinoma invading the deep submucosa (Fig. 1) [3]. Lymph node metastasis and lymphatic or blood vessel invasion are uncommon in m_1 or m_2 carcinoma and occur after progression to m_3 or sm_1 disease [1,3,4]. In sm_2 and sm_3 carcinoma, lymph node, lymphatic, and vascular involvement occur more frequently than in less advanced tumors, leading to a poor prognosis [4].

Various monoclonal antibodies have been developed and used to monitor the cell cycle and the proliferative activity of cancer cells [5,6]. MIB-1 is a mouse monoclonal antibody raised against part of the Ki-67 antigen encoded by a 100 bp fragment containing 66 bp repetitive elements of Ki-67 cDNA [7]. MIB-1 recognizes the Ki-67 antigen, which is expressed exclusively in the nuclei of proliferating cells (i.e., cells in the G1, S, G2, and M phases) [7]. Before the development of MIB-1, monoclonal antibodies to the Ki-67 antigen had to be used with fresh frozen sections. However, MIB-1 can be applied to paraffin-embedded sections after heating to invigorate the target antigen [8,9].

In the present study, the proliferative activity of superficial esophageal carcinoma was assessed by immunostaining with the monoclonal antibody MIB-1 and computer image analysis. The proliferative activity of tumor cells was analyzed in relation to the depth of tumor invasion and the location within a single tumor focus. The relationship of proliferative activity to pathological prognostic factors, including histological differentiation and lymph node metastasis, lymphatic and vascular involvement, was investigated in order to determine the prognostic value of immunopositivity for MIB-1.

TABLE I. Histopathologic Characteristics in the Cases With Superficial Squamous Cell Carcinoma of the Esophagus*

Depth of invasion	No. of cases	Histologic classification			n		ly		v	
		Wel.	Mod.	Por.	(–)	(+)	(–)	(+)	(–)	(+)
m_1	5	—	—	—	5	0	5	0	5	0
m_2	4	—	—	—	4	0	4	0	4	0
m_3	5	—	—	—	4	1	1	4	3	2
sm_1	7	0	5	2	4	1	2	5	7	0
sm_2	8	2	4	2	2	6	2	6	6	2
sm_3	9	3	5	1	5	4	3	6	5	4

*Wel., well-differentiated; Mod., moderately differentiated; Por., poorly differentiated; n, lymph node metastasis; ly, lymphatic invasion; v, blood vessel invasion; –, absent; +, present; m, mucosal cancer; sm, submucosal cancer.

MATERIALS AND METHODS

Materials

Specimens of superficial squamous cell carcinoma of the esophagus resected from 38 previously untreated patients at the Second Department of Surgery of Tokai University from 1985 to 1994 were studied. All of the specimens were subjected to full histopathological evaluation, which showed a uniform depth of invasion on consecutive paraffin-embedded sections and confirmed the presence of a sufficient number of tumor cells for the present study. The depths of superficial carcinoma were as follows: m_1 in five patients, m_2 in four patients, m_3 in five patients, in seven patients, sm_2 in eight patients, and sm_3 in nine patients. The histopathological characteristics (histological differentiation, lymph node metastasis, and lymphatic or blood vessel invasion) of the carcinomas are shown in Table I. Histological typing was only performed for sm_1, sm_2, and sm_3 carcinoma because it is sometimes difficult for mucosal carcinoma. All the specimens were fixed in 10% formalin and embedded in paraffin.

Immunostaining for the Ki-67 Antigen

Each paraffin-embedded specimen was cut into 5 μm sections, which were dewaxed, washed with 0.01 M phosphate-buffered saline (PBS), and heated in 0.01 M citrate-buffer, pH 6.0, by a microwave processor (H2500, Energy Beam Sciences Inc., Agawam, MA) for 20 minutes (10 minutes twice at 100°C) to retrieve the Ki-67 antigen. In the microwave, the sections were kept in a plastic staining jar suitable for heating. Then the sections were treated with 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity. After washing with PBS, normal sheep serum (Cosmo Bio Co. Ltd., Tokyo, Japan) was added to block nonspecific reactions. A 100-fold dilution of the monoclonal antibody MIB-1 (Immunotech S.A., Marseille Cedex, France) in PBS containing 1% serum albumin was applied and incuba-

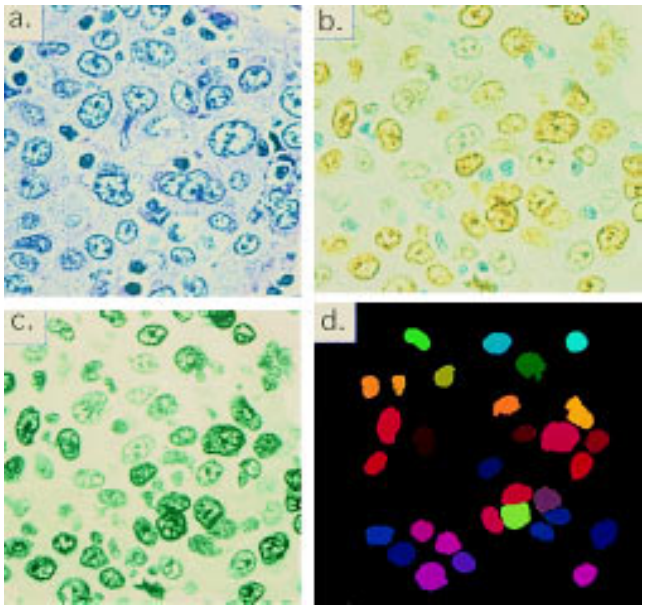

Fig. 2. Superficial esophageal carcinoma: computed image analysis. **a:** Tumors cells of squamous cell carcinoma of the esophagus counterstained with hematoxylin-eosin (40×). **b:** Brown reactive products in tumor nuclei of MIB-1 antibody. **c:** The corresponding nuclei stained positively with MIB-1 recognized by computed image analysis (black nuclei). **d:** Image analysis measuring the percentage of the corresponding nuclei (some coloration).

tion was performed for 1 hour at room temperature. After washing with PBS, immunostaining was done by the avidin-biotin complex (ABC) technique using biotin-labeled anti-mouse IgG (Amersham International plc., Buckinghamshire, England) and the peroxidase-labeled streptoavidin biotin (LSAB) method (DAKO A/S, Copenhagen, Denmark). After color was developed with 3,3-diaminobenzidine, counterstaining was done with hematoxylin and eosin.

Assessment of Ki-67 Expression

Ki-67 expression was evaluated using a computer image analyzer (Vidas, Carl Zeiss, Jena, Germany). Individual tumor cells were identified by microscopic observation at a magnification of 40×.

The site for assessment of proliferative activity was selected by examining hematoxylin-eosin stained sections (Fig. 2a). Using the adjacent serial section, tumor cells with nuclei containing brown reaction products were counted as positive (Fig. 2b) and these cells were selectively recognized by the image analyzer (Fig. 2c). Overlapping nuclei were counted separately by determining boundaries (Fig. 2d) and at least 1,000 nuclei were counted for each patient. The MIB-1 labeling index (LI) was calculated as the number of positive nuclei per 1,000 nuclei counted.

The growing edge of the carcinoma is defined as the invasive tip of the tumor. Proliferative activity was assessed at the invasive tip for the mucosal carcinomas and at both the invasive tip and the core of the nest for the submucosal carcinomas (Fig. 1). This was done because tumor growth largely depends on the proliferative kinetics at the invasive tip, as well as to assess the relationship between tumor growth and differentiation in larger lesions which usually show some degree of central keratinization.

Determination of the Prognostic Value of the LI

The relationships between the LI of MIB-1 and various histopathological prognostic factors were assessed. The histological depth of invasion was compared with the LI at the invasive tip for mucosal carcinoma and with the LI at the invasive tip and the core for submucosal carcinoma. The LI of MIB-1 was also compared with tumor differentiation, lymph node metastasis, lymphatic invasion, and blood vessel invasion.

Histopathological studies were performed in accordance with the *Japanese Guidelines for Clinical and Pathological Studies of Carcinoma of the Esophagus* [10] using sections stained with hematoxylin-eosin and Victoria blue hematoxylin-eosin stains.

Statistical Analysis

Data are expressed as the mean ± standard deviation (SD) and were tested for significance using Student's *t*-test. Differences were considered to be significant at $P < 0.05$.

RESULTS

LI and Histological Invasion

The LI of MIB-1 at the invasive tip of the tumor was 24.2 ± 5.6 for m_1 carcinoma, 22.3 ± 3.1 for m_2 carcinoma, 45.9 ± 5.8 for m_3 carcinoma, 34.6 ± 10.8 for sm_1 carcinoma, 45.8 ± 12.1 for sm_2 carcinoma, and 37.2 ± 11.9 for sm_3 carcinoma. The LI for m_3 carcinoma was significantly higher than that for m_1 and m_2 carcinoma, but was not significantly different from the values for sm_1, sm_2, or sm_3 carcinoma (Table II). The mean LI values for m_1/m_2 carcinoma, m_3/sm_1 carcinoma, and sm_2/sm_3 carcinoma were also calculated and compared. The mean LI for m_1/m_2 carcinoma (23.4 ± 4.7) was significantly lower than that for m_3/sm_1 carcinoma (38.2 ± 11.2) or that for sm_2/sm_3 carcinoma (41.2 ± 12.7). The mean LI for m_1/m_2 carcinoma was also significantly lower than that for m_3 carcinoma plus submucosal carcinoma (39.9 ± 12.2) (Table II).

LI Values at Different Tumor Sites

The LI values obtained from the invasive tip and the core of submucosal carcinoma were compared. The LI at the core was 21.6 ± 15.3 for sm_1 carcinoma, 28.5 ± 11.7 for sm_2 carcinoma, and 25.9 ± 13.1 for sm_3 carcinoma. The LI at the invasive tip was significantly higher ($P <$

TABLE II. Correlation Between Depth of Invasion and MIB-1 LI in the Invasive Tip of Tumor in Superficial Squamous Cell Carcinoma of the Esophagus

Depth of invasion	No. of cases	LI (%)
m_1	5	24.2 ± 5.6
m_2	4	22.3 ± 3.1
m_3	5	45.9 ± 5.8
sm_1	7	34.6 ± 10.8
sm_2	8	45.8 ± 12.1
sm_3	9	37.2 ± 11.9
m_1–m_2	9	23.4 ± 4.7
m_3–sm_1	12	38.2 ± 11.2
sm_2–sm_3	17	41.2 ± 12.7
m_3–sm_3	29	39.9 ± 12.2

*$P < 0.01$.

TABLE III. Superficial Esophageal Carcinoma: Correlation Between Portion of Cancer and MIB-1 LI in Submucosal Cancer

Depth of invasion	No. of cases	Labeling index (%) Tip		Core	
sm_1	7	34.6 ± 10.8		21.6 ± 15.3	($P = 0.091$)
sm_2	8	45.8 ± 12.1	—**—	28.5 ± 11.7	
sm_3	9	37.2 ± 11.9		25.9 ± 13.1	($P = 0.073$)
sm_1 – sm_3	24	38.7 ± 12.8	—*—	25.5 ± 12.5	

*$P < 0.01$.
**$P < 0.05$.

0.05) for sm_2 carcinoma and tended to be higher for sm_1 and sm_3 carcinoma ($P = 0.091$, $P = 0.073$, respectively). The mean LI at the invasive tip was also significantly higher ($P < 0.01$) than that in the core for submucosal carcinoma overall (Table III).

LI and Tumor Differentiation

The degree of differentiation was determined in 24 patients with submucosal carcinoma and its relationship to the LI of MIB-1 was studied. The LI at the invasive tip was 38.5 ± 9.9 for well-differentiated carcinoma (n = 5), 33.9 ± 12.4 for moderately differentiated carcinoma (n = 14), and 52.3 ± 3.4 for poorly differentiated carcinoma (n = 5). The corresponding values for the core of the tumor were 25.0 ± 9.2, 18.4 ± 8.7, and 45.9 ± 6.2. For moderately differentiated carcinoma, the LI at the invasive tip was significantly higher ($P < 0.01$) than that in the core. A trend towards a higher LI ($P = 0.055$) at the invasive tip was also noted for well-differentiated carcinoma, while no apparent difference was observed for poorly differentiated carcinoma. The LI values at both the invasive tip and the core of poorly differentiated carcinoma were significantly higher ($P < 0.05$) than the respective values for well or moderately differentiated carcinoma (Table IV).

LI and Lymph Node Metastasis

The LI values in the presence and absence of lymph node metastasis were studied in m_3 and submucosal carcinomas which were considered to be potentially associated with nodal involvement. The LI at the invasive tip was significantly higher ($P < 0.05$) for submucosal carcinomas with lymph node metastasis (n+) than for submucosal carcinomas without lymph node metastasis (n−) (43.3 ± 10.6 vs. 33.3 ± 13.1). However, when the cases with m_3 carcinoma were added, the LI at the invasive tip did not differ significantly ($P = 0.191$) between the tumors with and without nodal metastasis (43.0 ± 10.2 vs. 37.1 ± 13.2) (Table V).

LI and Lymphatic Invasion

The LI values in the presence and absence of lymphatic invasion were also studied in m_3 and submucosal carcinoma. The LI at the invasive tip of submucosal carcinoma with lymphatic invasion (ly+) was significantly higher ($P = 0.020$) than that for submucosal carcinomas without lymphatic invasion (ly−) (42.8 ± 11.7 vs. 30.4 ± 10.8). Even after adding the cases with m_3 carcinoma, the LI at the invasive tip was still significantly higher ($P < 0.05$) for the tumors with lymphatic involvement (43.2 ± 14.36 vs. 32.8 ± 12.1) (Table V).

LI and Blood Vessel Invasion

Finally, the LI values in the presence and absence of blood vessel invasion were compared for m_3 and submucosal carcinoma. The LI at the invasive tip was 37.3 ± 12.8 for submucosal carcinoma without blood vessel invasion (v−) and 42.8 ± 12.0 for submucosal carcinoma with blood vessel invasion (v+) (not significant, $P = 0.365$). For m_3 and submucosal carcinoma combined, the LI at the invasive tip was 39.0 ± 12.7 in the absence of blood vessel invasion and 39.0 ± 10.5 with blood vessel invasion, showing no significant difference ($P = 0.449$) (Table V).

DISCUSSION

The Committee for Clinical Typing of Superficial Esophageal Carcinoma of the Japanese Society for Esophageal Diseases has proposed that superficial esophageal carcinoma should be classified into six types (m_1–sm_3) based on the depth of invasion [10]. Many investigators have suggested that the depth of invasion is closely related to the incidence of lymph node and vascular involvement and is an important factor to be considered in determining the therapeutic approach [3,4]. Mucosal carcinoma classified as m_1–m_2 is rarely associated with lymph node metastasis and is usually treated by endoscopic mucosal resection [3,4,11,12], which is less invasive than open surgery. However, esophageal carci-

TABLE IV. Correlation Between Histologic Classification of Differentiation and MIB-1 LI in Submucosal Cancer

Histologic classification	No. of cases	LI (%) Tip	LI (%) Core
Well differentiated	5	38.5 ± 9.9	25.0 ± 9.2
Moderately differentiated	14	33.9 ± 12.4	18.4 ± 8.7
Poorly differentiated	5	52.3 ± 3.4	45.9 ± 6.2

*$P < 0.01$.
**$P < 0.05$.

TABLE V. Correlation Between MIB-1 LI at the Invasive Tip and Lymph Node Metastasis, Lymphatic Invasion, and Blood Vessel Invasion*

Depth of invasion	Histopathologic characteristics		No. of cases	LI (%)	*P* value
$sm_1 - sm_3$	n	Absent	11	33.3 ± 13.1	0.050
		Present	13	43.3 ± 10.6	
	ly	Absent	8	30.4 ± 10.8	0.020
		Present	16	42.8 ± 11.7	
	v	Absent	15	37.3 ± 12.8	0.365
		Present	14	42.8 ± 12.0	
$m_3 - sm_3$	n	Absent	15	37.1 ± 13.2	0.191
		Present	14	43.0 ± 10.2	
	ly	Absent	9	32.8 ± 12.1	0.028
		Present	20	43.2 ± 10.8	
	v	Absent	8	39.0 ± 12.7	0.449
		Present	21	39.0 ± 10.5	

*n, lymph node metastasis; ly, lymphatic invasion; v, blood vessel invasion.

noma with deeper invasion (m_3–sm_3) requires radical resection by thoracotomy and laparotomy. In the present study, the proliferative behavior of superficial esophageal carcinoma was investigated by labeling proliferating cells with an anti-human Ki-67 monoclonal antibody (MIB-1). The relationship of proliferative activity to various histopathological factors was investigated to assess the predictive value of the LI for MIB-1 in superficial esophageal carcinoma.

Proliferating cells are in G1, S, G2, or M phase of the cell cycle, while nonproliferating cells are in G0 phase. In 1982, a monoclonal antibody to bromodeoxyuridine, a homologue of thymidine, was used for immunohistochemical staining of S phase cells [6,13]. In 1983, Gerdes et al. [14,15] developed a monoclonal antibody that reacted with the Ki-67 antigen. The antibody can identify proliferating cells (i.e., cells in G1, S, G2, and M phases) by recognizing this antigen, which is exclusively expressed in the nuclei of such cells. Ki-67 immunostaining has already been widely applied to the grading of breast cancer [16], lung cancer [17], colorectal carcinoma [18], gastric cancer [19], and hepatocellular carcinoma [20], and the prognostic significance of this marker has been well documented. However, the application of this technique is restricted to fresh frozen sections. Proliferating cell nuclear antigen is another commonly used proliferation marker that accumulates in late G1 phase and S phase cells [21,22]. Proliferating cells can be detected in paraffin-embedded sections by immunostaining for this antigen [23]. However, the application of this method to quantitative studies is limited by variation in the phases of the cell cycle identified depending on the method of fixation [24] and by unstable staining which impairs distinguishing between positive and negative cells [9]. In contrast, the MIB-1 antibody that we used in the present study can be applied to paraffin-embedded sections after heating to enhance the antigenicity of Ki-67. Immunostaining with MIB-1 has a high reproducibility as the staining pattern is independent of the method of fixation and shows little variability [9]. Therefore, MIB-1 appears to be suitable for the quantitative assessment of cell proliferation in superficial esophageal carcinoma.

In the normal esophagus, immunostaining with MIB-1 is seen exclusively in the basal layer of the epithelium, while the superficial layer is not stained. Cells in the superficial layer are better differentiated, parakeratinized, and have lost their nuclei, while the basal layer consists of proliferating cells [25]. Therefore, Ki-67 expression may be closely related to the proliferative activity of esophageal cells. A previous study showed that the LI of superficial esophageal carcinoma increased as tumor invasion became deeper, with the increase being significant for carcinoma invading the muscularis mucosa [26]. Another study did not demonstrate any significant association of the LI with the depth of invasion in more advanced esophageal carcinomas [27]. In the present study, the LI at the invasive tip of m_3 carcinoma was significantly higher than for m_1 or m_2 carcinoma. Carcinoma m_1 and m_2, for which endoscopic mucosal resection is recommended, showed a lower proliferative activity compared to more advanced carcinoma. This may partly explain why the cases of m_1 and m_2 carcinoma are generally without lymph nodal, lymphatic, or vascular involvement. The low proliferative activity of m_1 and m_2 carcinoma also suggests that slow growth may occur over the long term, which is consistent with the common endoscopic findings in patients with such carcinomas [28]. The significantly higher proliferative activity of m_3 carcinoma compared to m_1 or m_2 carcinoma

may contribute to the increase of lymph node, lymphatic, or blood vessel invasion due to accelerated tumor growth. These results suggest that m_3 carcinoma may be borderline between treatments by endoscopic surgery or open surgery and should be further studied in more detail in the future.

For all three types of submucosal carcinoma (sm_1–sm_3) and for submucosal carcinoma overall, the LI at the invasive tip was higher than that of the core. Esophageal carcinoma usually undergoes differentiation and keratinization towards the center of the tumor focus. This is supported by our finding that the invasive tip showed significantly higher proliferative activity than the core of these tumors.

Proliferative activity was also analyzed in relation to tumor differentiation. The LI at the invasive tip of moderately differentiated carcinoma was significantly higher than that in the core, and a similar trend was observed for well-differentiated carcinoma. In contrast, proliferative activity did not differ significantly between the invasive tip and the core of poorly differentiated carcinoma. In both regions, the proliferative activity of poorly differentiated carcinoma was significantly higher than that of well and moderately differentiated carcinoma. Thus, tumor cells in the core of poorly differentiated carcinoma had a higher proliferative activity compared to the corresponding region of well or moderately differentiated carcinoma. Concerning the relationship between Ki-67 expression and tumor differentiation, some authors have found no difference of LI in relation to the degree of differentiation [27,29], while others have reported a higher positive rate in poorly differentiated carcinoma compared to well or moderately differentiated carcinoma [30]. Although some carcinomas show variation in differentiation at different sites (i.e., heterogeneity), it may be accepted that proliferative activity has an inverse correlation with the degree of differentiation, which is supported by the results of the present study.

Regarding the relationship between proliferative activity and lymph node metastasis, the percentage of Ki-67-positive tumor cells correlated with nodal involvement in breast cancer [31]. Studies on esophageal carcinoma have also shown that the LI of MIB-1 differs significantly between tumors with and without lymph node metastasis [26,29]. In the present study, the LI of submucosal carcinomas with lymph node metastasis was significantly higher than that of tumors without nodal involvement. However, the difference between m_3 and submucosal carcinomas with or without lymph node metastasis did not reach significance, probably due to the high proliferative activity of some m_3 carcinomas without nodal involvement. It has been reported that significant prognostic factors for esophageal carcinoma include the presence of lymph node involvement and the number of the metastatic nodes [32]. Therefore, the LI for MIB-1 may be a useful prognostic factor when determining the therapeutic approach for esophageal carcinoma, since a high LI value may be predictive of lymph node metastasis.

The LI data for carcinomas with and without lymphatic or blood vessel invasion were analyzed in a similar way. A previous study showed that positivity for MIB-1 had no significant correlation with the degree of lymphatic or blood vessel invasion [29]. In the present study, the LI of tumors with lymphatic invasion was significantly higher than that of tumors without it, although no significant difference was observed between tumors with and without blood vessel invasion. This suggests that a high proliferative activity is predictive of lymphatic involvement and that the LI of MIB-1 may be a useful prognostic factor.

In conclusion, Ki-67 expression as identified by immunostaining with monoclonal antibody MIB-1 may indicate the grade of malignancy of superficial esophageal carcinoma. Immunostaining of biopsy specimens with MIB-1 may allow preoperative histological grading of esophageal carcinoma and provide a useful guide to the selection of therapy. In addition, the immunostaining of resected carcinomas may be helpful for estimating the possibility of recurrence and for determining the need for postoperative adjuvant therapy.

CONCLUSIONS

The correlation between histopathological characteristics and the proliferative activity of superficial esophageal carcinoma was investigated. Proliferative activities of cancer cells in superficial esophageal squamous cell carcinoma and immunostaining with the MIB-1 were statistically significantly related to the depth of invasion, differentiation, lymph node metastasis, and lymphatic invasion.

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